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EFFECTS OF DIFFERENT MEDIATORS ON FIBROBLAST PROLIFERATION

A.M.E.NOURI,I.M.LEIGH,J.MANGERA,A.SHAMSA* AND H.FESTENSTEIN

*From the Department of Immunology and Experimental Dermatology, The London Hopital, London E1 2AD, and the *Mashhad University of Medical Sciences, Mashhad, Islamic Republic of Iran.*

ABSTRACT

In this study, we have investigated the proliferative response of human dermal fibroblasts isolated from normal and psoriatic individuals to different cytokines. We found that IL-1, TNF (α) and EGF are stimulatory whereas IFN α and γ both showed inhibitory activity on fibroblast proliferation. IL-6 a more recently described cytokine, showed no detectable effects on cell proliferation. Investigating the combined effects of these mediators on fibroblasts, we found that while EGF has additive effects on IL-1 or TNF (α) activated cells, both IFNs showed suppressive activity on the cell proliferation. There was no statistical difference in proliferative response of fibroblasts isolated from normal and psoriatic individuals. The significance of these findings is discussed.

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INTRODUCTION

Synovial fibroblast proliferation is an early characteristic feature of synovitis associated with rheumatoid arthritis. The hyperproliferation of these cells contribute to excess collagenase and prostaglandin (PGE2) production which are the most likely mediators involved in tissue damage and remodelling. The proliferation of fibroblasts is not only associated with tissue damage but is also characteristic of granuloma formation and the overall process of fibrosis. This often accompanies the pathogenesis of many inflammatory and infectious diseases including tuberculosis and schistosomiasis where extensive granulomatous and fibrotic reactions take place.

In parallel to fibroblast proliferation there is an infiltration of mononuclear cells (MNC) into the affected area.[1] This has led many investigators to suggest that MNC and their products contribute to fibroblast stimulation and eventual proliferation. Thus Dayer, Krane, Russell et.al.[2] have demonstrated that a factor produced by activated MNCs and designated "Mononuclear Cell Factor" is capable of activating PGE2 and collagenase release from synovial fibroblasts of rheumatoid patients. Synovial fibroblasts have also been shown to respond to interferons by producing another mediator of inflammation namely hyaluronic acid in culture.[3] A factor produced by MNC capable of inducing cartilage martix degradation via chondrocyte activation has been reported by others.[4] Using a well defined system Schmidt, Mizel, Cohen et.al.[5] have demonstrated the presence of a factor in a mixed lymphocyte culture capable of stimulating human dermal fibroblasts to proliferate. These authors have shown very convincingly that this factor has interleukin-1 (IL-1) activity. Hart, Powell, Cooksley et.al.[6] described a factor produced by MNC capable of inhibiting collagen synthesis by fibroblasts. It is interesting to mention that a number of reports have also indicated that fibroblasts isolated from affected areas like psoriatic lesions and plaques in many ways behave differently from those of normal fibroblasts.[7-12]

In the more recent years many workers have focused their attention on how different biological mediators exert their effects in concert. Thus interferon and tumour necrosis factor (TNF) have been shown to synergise in destroying malignant tumour cells isolated from human breast cancer.[13] Wong and Doddel[14] have

Correspondence: AME Nouri, Department of Immunology, The London Hospital, London E1 2AD.

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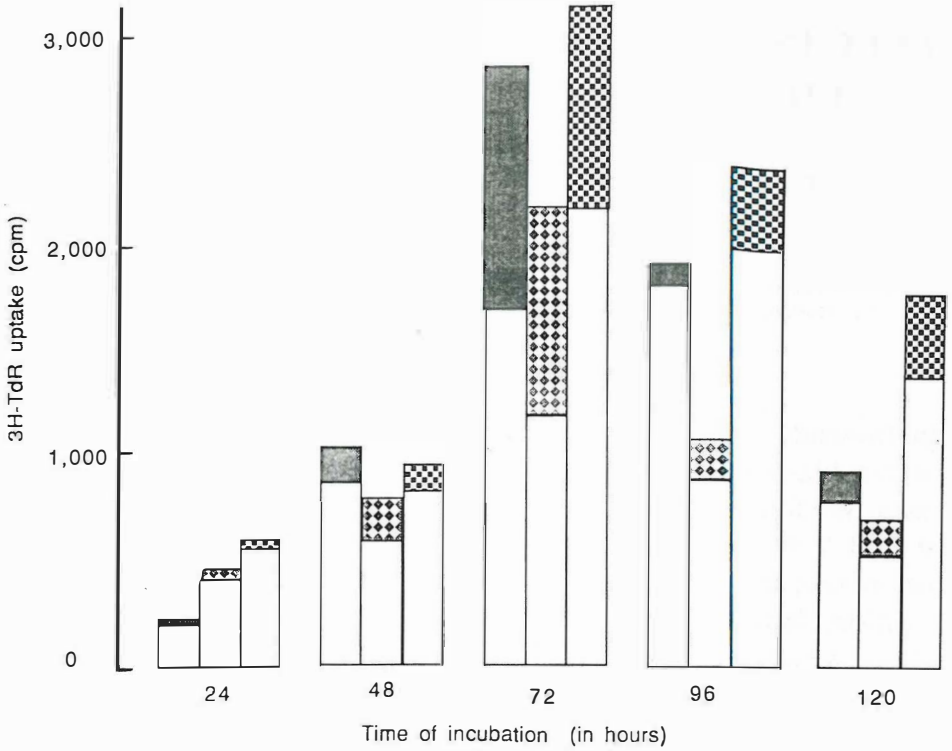


Fig 1. Time-course of fibroblast proliferation of three individuals in the presence (shaded parts of the columns) and absence of (empty parts of the columns) IL-1 (0.01 u/ml). The results are expressed in mean+1SD of 3H-TdR uptake.
The different shaded columns indicate the response of fibroblasts of different individuals.

shown the synergistic effects of TNF (α) and interferon (IFN) in inhibiting viral replication. Using IL-1 and TNF Bevilacque, Pober and Majean et.al.[15] found that IL-1 and TNF both induce the expression of new proteins on the cell surface of endothelial cells and this effect becomes additive when the two mediators are added in combination. The aim of this project using a proliferative assay is to investigate: 1) the effect of the pure or recombinant form of different mediators on human skin fibroblasts, 2) how fibroblasts are affected by the combination of these mediators and, 3)whether there is any difference in the way fibroblasts isolated from psoriatic lesions and normal skin respond to these mediators.

MATERIALS AND METHODS

Fibroblast separation and culture

Fibroblasts were prepared by a modified technique described by Dayer et.al.[2] Briefly, skin biopsies obtained from skin of normal individuals and psoriatic patients were cut to small fragments. After washing, 10 ml aliquot of trypsin-EDTA (O.5 g trypsin 1:125 and 2g of EDTA) was added and incubated for 2hrs at 37°C with frequent agitation. After the incubation, cell suspensions were passed through gauze and the larger fragments i.e dead tissues were discarded. Each single cell suspension was washed twice in RPMI containing 10% heat inactivated foetal calf serum (FCS, Gibco) and cultured in flask (Falcon). Once confluence reached, the cells were trypsinised (5 to 10 mins at 37°C) and after washes, the cell pellet was resuspended in RPMI plus 10% FCS.

Aliquots of the cells were frozen at every subculture for later use (see results section). Fibroblasts were aliquoted into 96 well flat-bottomed microtitre plates (Falcon) at different cell numbers/well (see results section) and cultured for different lengths of time with or without mediators at 37°C in 5% CO_2 humidified incubator. Twenty four hours before the termination of the culture, O.1 uCi of tritiated thymidine (3HTdR, 5 mCi/mM,Amersham) was added to each well. The cells were harvested onto glass filter paper using a semiautomated cell harvester and the degree of 3H-TdR incorporation into cellular DNA was measured using standard scintillation fluid technique.

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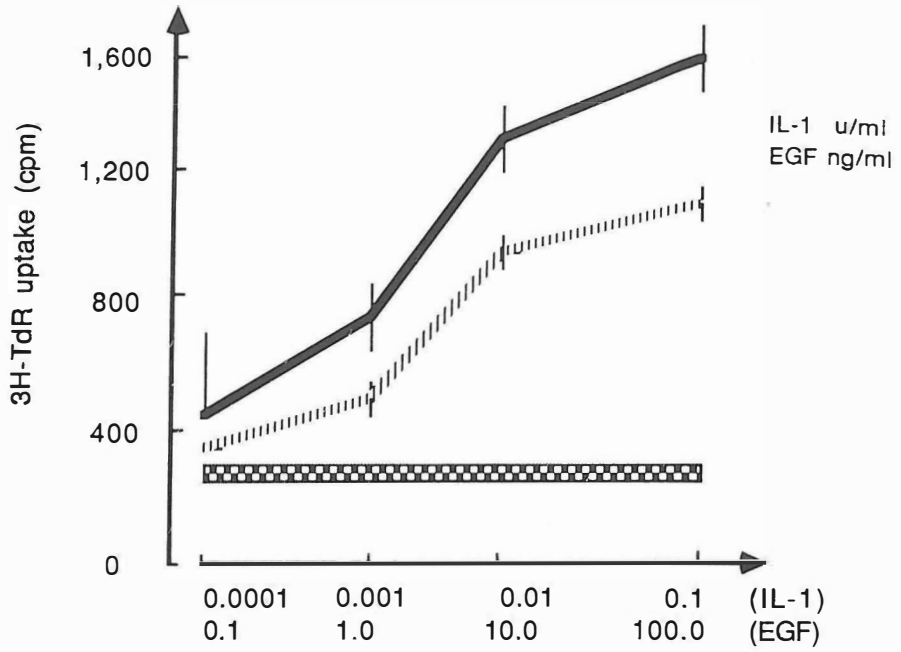


Fig 2. Fibroblast proliferation spontaneously (▩) and in response to IL-1 (▬) and EGF (▥). The results are expressed in mean+ 1SD of 3H-TdR uptake.

Mononuclear cell supernatant preparation

Mononuclear cells were prepared as described by Nouri, Panayi and Goodman.[16] Briefly, diluted peripheral blood from a volunteer was layered onto density gradient solution (Lymphoprep). After centrifugation for 20 mins at 2,000 rpm, the interface cells mainly consisting of MNC were aspirated, washed and resuspended in RPMI plus 10% FCS. The cells were dispensed into culture flasks to give two million cells/ml and treated with mitogen (PHA, 2ug/ml). After incubation for 2 hrs at 37°C, the cells were washed three times to get rid of the mitogen and culturing continued at the above cell number for 36 hrs. The cell-free supernatant was obtained and aliquoted and kept at-70°C until use.

MATERIALS

Phytohaemagglutinin (PHA)	Wellcome
Interleukin-l (IL-1)	Genzyme (recombinant)
Interferon (IFN, γ)	Biogen (recombinant)
Tumor necrosis factor (TNF)	Dr Meager (gift)
Interferon (IFN, α)	Dr Papadimitriou (gift)
Interleukin-6	Dr. Kishimto (recombinant).
Epidermal growth factor (EGF)	Sigma, (EGF)

All experiments were performed in three replicates for each treatment and statistical analysis was done using paired Student t-test.

RESULTS

In order to establish the optimal cell number for maximising cell proliferation, fibroblasts were aliquoted into 96 well microtitre plates to give 2,000, 5,000, 10,000 and 20,000 cells/well and were incubated at 37°C for 24hrs. After the incubation supernatants were replaced by medium containing 10% FCS (see below) and optimal concentration of IL-1 (see below). The incubation continued for a further 72hrs (see below) and the degree of cell proliferation was established as described in the Methods and Materials section.

It was found (results not shown) that 10,000 cells/well gives the maximum degree of 3H-TdR uptake and this cell number was used for all the experiments described in this study. In a similar experiment we established that 10% FCS gives (results not shown) near enough optimal degree of cell proliferation.

Time-course of fibroblast proliferaton

Having established the appropriate cell number and serum level, we concentrated on the time-course of cell division. Fibroblasts were aliquoted into microtitre plates and the optimal concentration of IL-1 (0.01 u/ml) was added and the plates were incubated for total of 24, 48, 72, 96 and 120 hrs. The degree of 3H-TdR uptake was measured, results of which are presented in Fig. 1. As can be seen all the three fibroblast cell lines showed increasing 3H-TdR uptake as the time of the incubation increased, reaching their maximum at 72 hrs followed by gradual decrease at 96 and 120 hrs.

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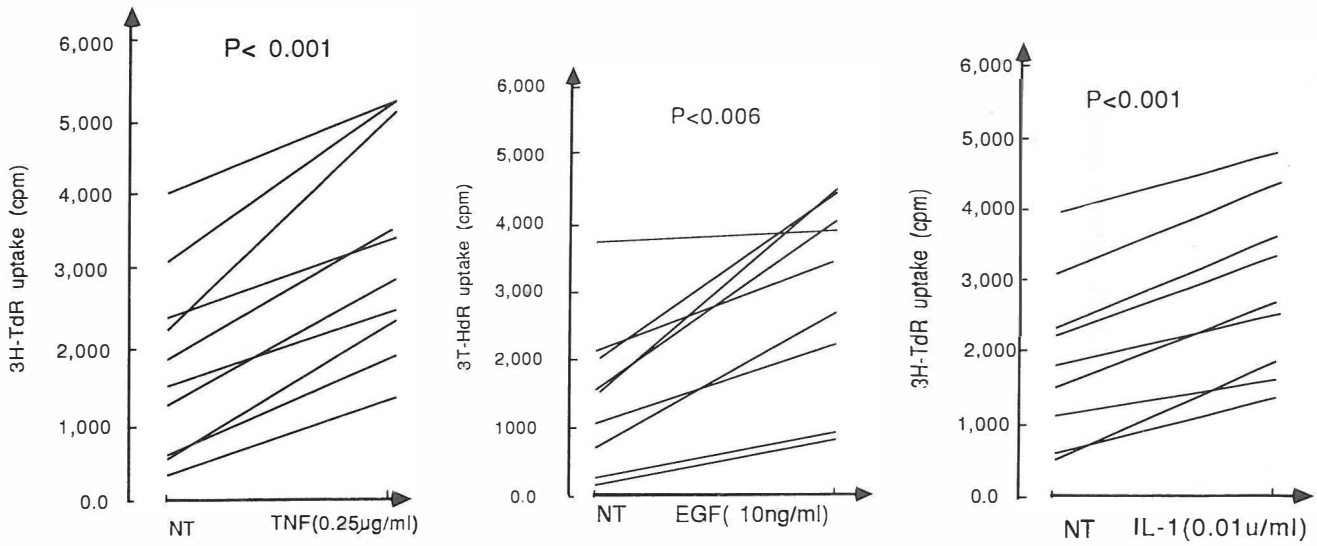


Fig 3. Proliferative reponse of fibroblasts of different individuals to (a) TNF, (b) EGF (c) IL-1 and (d) IFN. The results are expressed in mean+1SD of 3H-TdR uptake.

Although there is a difference between IL-1 treated and untreated cells at all time intervals investigated, the greatest difference was seen at 72 hrs and this was chosen to be the optimal time of incubation and was used for the experiments described in this study.

Fibroblast sub-culturing

Our next investigation was to establish whether sub-culturing of fibroblasts, i.e trypsinising cells and expanding them on repeated intervals, influence their sensitivity to soluble mediators like IL-1. Fibroblasts of three individuals which were collected after each sub-culturing and kept in liquid nitrogen were thawed out and grown in microtitre plates as before. The results (not shown) demonstrated that subculturing of the cells to as many as 20 times over a period of six months does not influence their sensitivity to IL-1.

Dose response of fibroblasts to soluble mediators

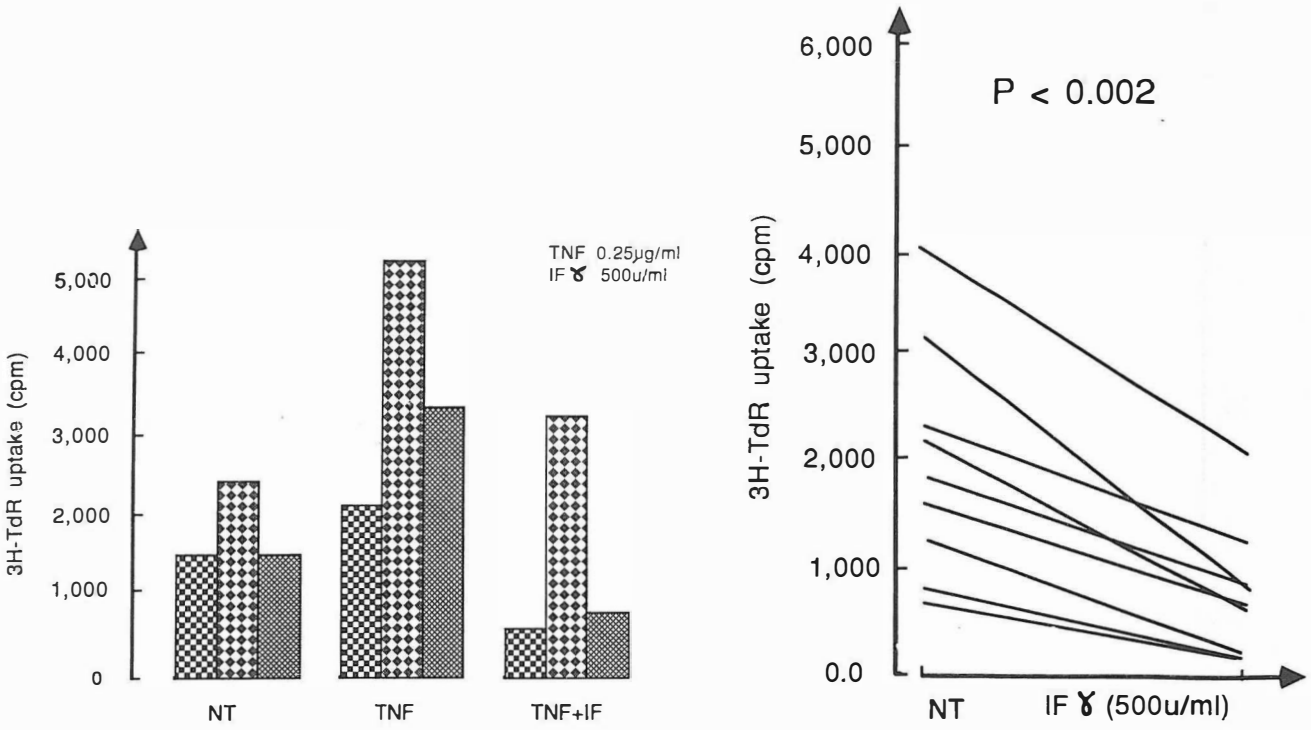
Dose response curve for all the mediators included in this study was investigated and concentration for each to achieve optimal cell proliferation was established. Results for IL-1 and EGF are presented in Fig.2. As can be seen IL-1 as low as 0.001 u/ml induces a significant increase in cell proliferation over the background of cell division. This difference becomes greater as the concentration of IL-1 increases reaching its optimum at 0.01 u/ml. Further increase in IL-1 levels to 0.1 u/ml resulted in the slowing down of cell proliferation. It is possible that when 0.1 u/ml of IL-1 is added to the fibroblast culture, they are stimulated to produce an excessive amount of arachidonic acid metabolite $PGE2^2$ which in turn might be down-regulatory for cell division. This possibility was confirmed using cyclooxygenase inhibitor, i.e indomethacin (0.1 ug/ml). It was found that (results not shown) the addition of this drug to IL-1 (0.1 u/ml) increased proliferation of cells by at least 40%.

EGF, a known fibroblast mitogen, showed a similar pattern of activity to that of IL-1 giving optimal proliferative activity at 10 ng/ml. This approach enabled us to establish the optimal concentration for all the mediators included in this study. They are IL-1= 0.01 u/ml, TNF= 0.25 ng/ml, EGF= 10 ng/ml, IFN γ = 500 u/ml and IFN α = 500 u/ml. Using these concentrations, fibroblasts isolated from different individuals were investigated. As can be seen from Fig. 3a, all the ten cell lines treated with TNF showed increased proliferation over the untreated cell, increasing the mean of 3H-TdR uptake from 1, 859±1,204 to 3,352±1,400 with $p<0.001$. EGF (3b) stimulated 8 of 9 fibroblasts increasing the mean from 1,493±1, 073 to 2,927±1,954 with $p< 0.006$. IL-1 (3c) like TNF stimulated all the cell lines increasing the mean from 2,022±1,154 to 2,854±1,119 with $p<0.001$. Both interferons on the other hand suppressed the cell proliferation. In the case of IFN γ (3d) all the individual lines tested showed inhibition decreasing the mean of 3H-TdR uptake from 2,028±1,158 to 861±631 with $p<0.002$. The investigation of Il-6 however on fibroblasts showed no detectable activity (data not shown) excluding the possibility of autocrine activity of this mediator on these cells.

Combined effects of different mediators

In order to establish the combined effects of different mediators, fibroblasts were treated with the optimal concentrations of two different mediators and

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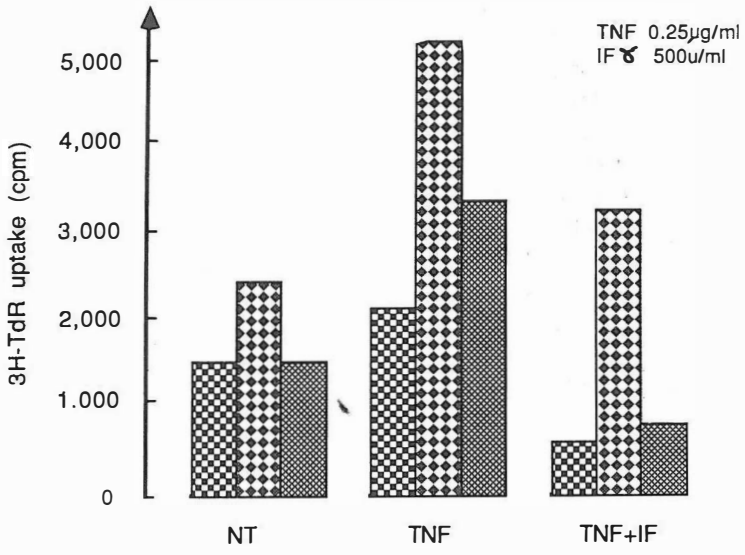


Fig 4. Proliferative response of fibroblasts of three different individuals (indicated in columns with different shades) spontaneously (NT), in response to TNF alone and in reponse to TNF plus EGF (a) and TNF plus IFN (b). The results are expressed in mean +1SD of 3H-TdR uptake.

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